Chapter 2

Materials and Methods

2.1 MATERIALS

2.1.1 Media, reagents, chemicals and antibodies

Media for cell culture (DMEM and Ham's F12) and foetal bovine serum (FBS) were obtained from Gibco, Invitrogen (Carlsbad, CA, USA). Cell culture reagents such as Trypsin, Phosphate Buffered saline (PBS), Antibiotics, Glutamine, etc. were also obtained from Gibco, Invitrogen (Carlsbad, CA, USA). Chemicals for cell culture experiments Aphidicholin, Nocadazole, Polybrene, and Puromycin were obtained from Sigma (St Louis, MO, USA). Cyclosporine A, MTT (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide), wortmannin, UO126, SP 600125, cycloheximide, camptothecin, Tacrolimus/FK506 , Tween 20 and Malachite green were obtained from Sigma-Aldrich (St. Louis, MO, USA). Specific calcineurin substrate RII peptide, calmodulin, eIF-2α inhibitor salubrinal, MG-132 and caspase inhibitor z-VAD FMK were obtained from Calbiochem (San Diego, CA, USA). Cytotoxicity detection kit (LDH) was obtained from Roche Diagnostics, (Mannheim, Germany). Live /Dead cytotoxicity assay kit was obtained from Molecular probes, Life technologies, USA. Lipofectamine-2000 and Opti-MEM for transient transfections were also obtained from Invitrogen (Carlsbad, CA, USA).

Growth media for bacteria (LB) was obtained from HiMedia laboratories (Mumbai, India). Enzymes used for recombinant DNA experiments (Restriction endonucleases, DNA ligase) were obtained from New England Biolabs (Ipswich, MA, USA). Markers for DNA and protein gels were from Fermentas (Vilnius, Lithuania).

Various kits used for macromolecular isolation (Plasmid isolation kit-Mini and midi, Gel extraction kit, PCR purification kit, RNA isolation kit) were procured from Qiagen (Hilden, Germany) or HiMedia (India). Trizol reagent for RNA isolation was obtained from Invitrogen (Carlsbad, CA, USA). BCA protein estimation kit was from Pierce (Rockford Illinois, USA). Cell fractionation kit was obtained from Fermentas (USA). Kit for TUNEL assay kit was obtained from Invitrogen (Carlsbad, CA, USA).

PCR reagents (PCR buffer, dNTPs, $MgCl_2$, Taq DNA polymerase) were obtained from Fermentas. Polymerase for long PCRs (AccuTaq) was obtained from Sigma. Reverse transcriptase (SuperScript III) was obtained from Invitrogen. Various chemicals required

for preparation of regular buffers and solutions viz. Tris, Glycine, SDS, Sodium Chloride, Potassium Chloride, Disodium Phosphate, NP-40, Tween 20, Triton X 100, Formaldehyde, Glycerol, Agarose, Acrylamide, *Bis*- Acrylamide, Ammonium per sulphate (APS), TEMED, BSA, Propidium Iodide, RNase A etc. were obtained from Sigma (St Louis, MO, USA).

PVDF membrane, X – ray films and western blotting detection reagent (ECL prime) were obtained from GE Healthcare (Little Chalfont, UK). Protease inhibitor tablets were obtained from Roche (Penzberg, Germany). Anti mouse and anti-rabbit secondary antibodies tagged to HRP (Horse radish peroxidise) were obtained from Bangalore Genei (Peenya, India). Secondary antibodies for Immunofluorescence (anti mouse IgG and anti rabbit IgG) conjugated to Alexa Fluor (488 and 594) from Molecular Probes, Invitrogen and Vectashield mounting medium with DAPI was obtained from vector laboratories (Burlingame, CA, U.S.A).

Antibodies from different sources were used in the present study. The list of different antibodies used in the present thesis is provided in Table 2.1.

Table 2.1: List of antibodies used

S.No.	Antibody against	Antibody source	Catalogue no.	Antibody dilution used	
				Western Blotting	Immunoflu-orescence
1.	BiP	CST	3177	1:1000	1:200
2.	CHOP	CST	2895	1:1000	
3.	PDI	CST	3501	1:1000	
4.	IRE1α	CST	3294	1:1000	
5.	p-eIF2α	CST	3398	1:1000	
6.	eIF2α	Sigma	E0157	1:1000	
7.	PERK	CST	3192	1:1000	
8.	ATF6	Abcam	Ab37149	1:10000	
9.	p-SAPK/JNK	CST	4668	1:1000	
10.	SAPK/JNK	CST	9258	1:1000	
11.	Calnexin	CST	2679	1:1000	1:100
12.	Cyclophilin B	Abcam	Ab16045	1:10000	
13.	LC3B	CST	3868	1:1000	
14.	Cleaved Caspase 3	CST	9664	1:1000	
15.	Cleaved PARP	CST	9544	1:1000	
16.	Cyclin D1	CST	2978	1:1000	
17.	p27	Sigma	P2092	1:1000	
18.	p21	Sigma	P1484	1:1000	

19.	α-Tubulin	Sigma	T 6074	1:10000	1:200
20.	Lamin B	Santa Cruz	4503021	1:1000	
21.	GAPDH	Sigma	G9545	1:10000	
22.	Calcineurin	Sigma	C1956	1:1000	
23.	NFAT1	Abcam	Ab49161	1:1000	
24.	NFAT2	Abcam	Ab25916	1:1000	
25.	NFAT3	Abcam	Ab62613	1:1000	
26.	DSCR1 (RCAN1)	Sigma	D6694	1:1000	
27.	Ras	CST	3965	1:1000	
28.	p-Raf	CST		1:1000	
29.	p-AKT	CST	4060	1:1000	
30.	Alix	CST	2171	1:1000	
31.	Rabbit IgG (HRP)	Genei	621140380011730	1:10000	
32.	Mouse IgG (HRP)	Genei	621140680011730	1:10000	
33.	Goat IgG (HRP)	Sigma	A5420	1:10000	
34.	Rabbit IgG (Alexa 488)	Invitrogen		1:10000	
35.	Mouse IgG (Alexa 488)	Invitrogen		1:10000	

2.1.2 Buffers and solutions

I. Stock solutions

(a) HEPES pH 7.9

Components	Final concentration	For 100 ml
HEPES	1M	23.83g
H_2O		q.s

The pH was adjusted to 7.9 using 10M NaOH

(b) Potassium Chloride (KCl)

Components	Final concentration	For 100 ml
KCl	2M	14.91g
H_2O		q.s

(c) Sodium Chloride (NaCl)

Components	Final concentration	For 100 ml
NaCl	5M	29.22g
H_2O		q.s

(d) Ethylene Glycol Tetraacetic acid (EGTA), pH 7.0

Components	Final concentration	For 50 ml
EGTA	0.1M	1.902g
H_2O		q.s

The pH is adjusted to 7.0 using 10M NaOH

(e) Ethylenediamine tetraacetic acid (EDTA), pH 8.0

Components	Final concentration	For 500 ml
EDTA	0.5M	93.05g
H_2O		q.s

The pH is adjusted to 8.0 using 10M NaOH

(f) Dithiothreitol (DTT)

Components	Final concentration	For 5 ml
DTT	1.0M	0.7725g
H_2O		q.s

(g) NP-40

Components	Final concentration	For 10 ml
NP-40	10%	1ml
H_2O		9ml

(h) Leupeptin

Components	**Final concentration**	**For 1 ml**
Leupeptin (10mg/ml)	1mg/ml	50µl
H_2O		450 µl

(i) Aprotinin

Components	**Final concentration**	**For 1 ml**
Aprotinin (2mg/ml)	1mg/ml	500µl
H_2O		500µl

(j) Benzamidine

Components	**Final concentration**	**For 5 ml**
Benzamidine	250mg/ml	1.25g
H_2O		q.s

(k) Phenylmethylsulfonyl fluoride (PMSF)

Components	**Final concentration**	**For 5 ml**
PMSF	100mM	0.0871g
H_2O		q.s

(l) Acrylamide (29:1)

Components	**Final concentration**	**For 500 ml**
Acrylamide	29%	145g
***Bis*-acrylamide**	1%	5g
H_2O		q.s

(m) Ammonium persulfate (APS)

Components	Final concentration	For 10 ml
APS	10%	1g
H_2O		q.s

II. General Buffers

(a) Phosphate Buffered Saline (PBS)

Components	Final concentration	For 10X stock (1L)
Sodium Chloride (NaCl)	137 mM	80g
Potassium chloride (KCl)	2.7 mM	2g
Disodium phosphate (Na_2HPO_4)	10 mM	1.44g
Monopotassium phosphate (KH_2PO_4)	2 mM	0.24g

pH adjusted to 7.4 with HCl

(b) Tris Buffered Saline (TBS)

Components	Final concentration	For 10X stock (1L)
Tris Cl	50 mM	60.5g
Sodium Chloride (NaCl)	150 mM	87.6g

pH adjusted to 7.4 with HCl

III. For Immunoblotting

(a) Cell lysis buffer (RIPA Buffer)

Components	Final concentration
Tris pH 8.0	50 mM
NaCl	150 mM
NP-40	1%
Sodium deoxycholate	0.5%
SDS	0.1%
NaF	10mM
Sodium orthovanadate	1 mM
Aprotinin	1µg/ml
Leupeptin	1µg/ml
Pepstatin	1µg/ml

(b) 6X protein loading buffer (Laemmli buffer)

Components	Final concentration	For 100 ml
1.5 M Tris-Cl pH 6.8	60 mM	4 ml
SDS	2%	2g
Glycerol	10%	10 ml
β-mercaptoethanol	5%	5 ml
bromophenol blue	0.01%	0.01 g

(c) Stacking and resolving Acrylamide gels

Resolving gel (10 ml)

Components	For 8% gel (ml)	For 10% gel (ml)	For 12% gel (ml)
Water	4.6	4.0	3.3
30% acrylamide	2.7	3.3	4.0
1.5 M Tris (pH 8.8)	2.5	2.5	2.5
20% SDS	0.1	0.1	0.1
10% APS	0.1	0.1	0.1

TEMED	0.006	0.004	0.004

Stacking gel

Components	**For 10 ml**
Water	6.8 ml
30% acrylamide	1.7 ml
1.0 M Tris (pH 6.8)	1.25 ml
20% SDS	0.1 ml
10% APS	0.1 ml
TEMED	0.01 ml

(d) Running Buffer

Components	**Final concentration**	**For 10X stock (1L)**
Tris base	25 mM	30g
Glycine	192 mM	144g
SDS	0.1%	10g

(e) Transfer Buffer

Components	**Final concentration**	**For 10X stock (1L)**
Tris base	25 mM	30g
Glycine	192 mM	144g
Methanol	20%	200ml

(f) TBST

Components	**Final concentration**	**For 1L**
TBS		1000ml
Tween 20	0.1%	1ml

(g) Blocking Buffer

Components	Final concentration	For 100 mL
Fat free milk	5%	5g
TBST		Make up to 100ml

(h) Stripping Buffer

Components	Final concentration	For 1L
1.0 M Tris Cl (pH 6.7)	62.5 mM	62.5 ml
β-mercaptoethanol	100 mM	5.0 ml
SDS	2%	20 g

IV. For Immunofluorescence

(a) Fixative : 4% Formaldehyde

Components	Final concentration
Formaldehyde	4%
PBS	q.s.

(b) Permeabilization buffer: 0.2% Triton X100

Components	Final Concentration	For 10 ml
Triton X100	0.2%	0.02 ml
PBS	q.s	10 ml

(c) Blocking buffer: 2% BSA

Components	Final Concentration	For 10 ml
BSA	2%	0.2 g
PBS	q.s.	10 ml

V. For Cell fractionation

(a) Cytoplasmic extraction buffer (without protease inhibitors)

Components	Final Concentration	For 100 ml
1 M HEPES	10 mM	1 ml
2M KCl	10 mM	0.5 ml
0.5 M EDTA	0.1 mM	0.02 ml
0.1 M EGTA	0.1 mM	0.1 ml
H_2O		q.s

(b) Nuclear extraction buffer (without protease inhibitors)

Components	Final Concentration	For 100 ml
1 M HEPES	10 mM	1 ml
5M NaCl	0.2 M	4 ml
0.5 M EDTA	0.5 mM	0.1 ml
0.1 M EGTA	0.5 mM	0.5ml
H_2O		q.s

VI. For Electrophoretic mobility shift assay (EMSA)

(a) Polydeoxy (Inosinate-cytidylate) (Poly dI-dC)

Components	Final concentration	For 5 ml
Poly dI-dC (25 units)(1250µg)	1mg/ml	1250µg
H_2O		q.s

(b) Nuclear lysis buffer

Components	Volume from stock (µl)	Final concentration
Nuclear extraction buffer	974	
0.1M DTT	10	1mM
Leupeptin (1mg/ml)	2	2µg/ml
Aprotinin (1mg/ml)	2	2µg/ml
Benzamidine(250mg/ml)	2	0.5mg/ml
PMSF (100mM)	10	0.5mM

(c) EMSA Buffer

Components	Final concentration	For 1000 ml
Tris	0.25M	30.285g
Glycine	2M	150g
EDTA (0.5M)	0.01M	20ml
H_2O		q.s

The pH is adjusted to 8.5

(d) Binding Buffer (10X)

Components	Volume from stock (ml)	Final concentration
1M HEPES, pH 7.9	2	200mM
0.5M EDTA, pH 8.0	0.08	4mM
1M DTT	0.04	4mM
Sterile Glycerol	5	50%
Sterile H_2O	2.88	

VII. For Plasmid isolation

(a) Resuspension solution (Solution I)

Components	Final Concentration	For 100 ml
1 M Tris-HCl (pH 8.0)	50 mM	2.5 ml
EDTA	10 mM	2.0 ml
Glucose	50 mM	0.9 g

(b) Lysis solution (Solution II)

Components	Final Concentration	For 100 ml
10 M NaOH	0.2 M	2 ml
10% SDS	1%	10 ml

(c) Neutralization solution (Solution III)

Components	Final Concentration	For 100 ml
Potassium Acetate	3 M	29.4 g
Glacial acetic acid	11.5%	11.5 ml

VIII. For DNA electrophoresis

(a) TAE

Components	Final Concentration	For 50 X (1litre)
Tris	40 mM	242 g
Acetic acid	20 mM	57.1ml
0.5M EDTA	1 mM	100ml

(b) Agarose gel

Components	**0.8% gel.**	**1% gel**	**2% gel**
Agarose	0.8 g	1 g	2 g
TAE	100 ml	100 ml	100 ml

(c) DNA loading dye

Components	**Final Concentration**	**For 100ml**
Glycerol	30%	30ml
Bromophenol Blue	0.25%	0.25 g
Xylene Cyanol	0.25%	0.25 g

IX. For preparation of Ultra competent cells

Inoue buffer

Components	**Final Concentration**	**For 100ml**
0.5 M PIPES, pH 6.7	10 mM	2 ml
$CaCl_2.2H_2O$	15 mM	0.22 g
KCl	250 mM	1.865 g
$MnCl_2.4H_2O$	55 mM	1.088 g

pH adjusted to 6.7 with 1M KOH followed by filter sterilization.

X. For cell cycle analysis by flow cytometry

(a) Fixative

Components	**Final Concentration**	**For 100 ml**
Ethanol	35%	35 ml
Methanol	35%	35 ml

(b)DNA staining solution

Components	Final Concentration
Propidium Iodide (PI)	30µg/ml
RNase A	50 µg/ml

Volume is adjusted with 1X PBS

XI. Calcineurin phosphatase assay

(a)Cell lysis Buffer

Components	Final Concentration
Tris-HCl, pH 7.5	50 mM
Ascorbate	1 mM
DTT	1 mM
NP-40	0.02%
PMSF	50 µg/ml
Aprotinin	5 µg/ml
Pepstatin	5 µg/ml

(b)Reaction Buffer

Components	Final Concentration
Tris-HCl, pH 7.5	50 mM
NaCl	100 mM
$MgCl_2$	6 mM
DTT	0.5 mM
NP-40	0.025%
$CaCl_2$	0.5 mM
Ascorbic acid	5 mM

(c) Malachite green reagent

Components	Final Concentration
Ammonium molybdate	4.2%
Malachite green	0.45%
Tween 20	0.2%

2.2 Methods

2.2.1 Cell biology methods

2.2.1.1 Maintenance of cell lines

In the present thesis, various cell types were used for which the details are provided in the Table 2.2. SiHa, HeLa, HaCaT, U2OS, SaOs , A549, HPLD and HEK-293 cells were grown in Dulbecco's modified Eagle's medium (HyClone, Thermo Scientific, Logan, Utah, USA) supplemented with 2 mM glutamine (Gibco BRL), 100 U/ml penicillin and streptomycin (Gibco BRL, Carlsbad, CA, USA), and 10% fetal bovine serum (Gibco BRL, Carlsbad, CA, USA) under humified conditions at 37°C and 5% CO_2.

Cells were grown in cell culture dishes till they attained 70% confluency. For sub culturing, these were then trypsinised using 0.05% Trypsin EDTA solution and incubated for 5 minutes at 37^0C for cells to be detached from surface. The detached cells were then collected by gentle tapping the dish and pipetting. Trypsin was then inactivated by addition of FBS containing culture medium, transferred to a 15 ml tube and centrifuged at 1500 rpm for 2 minutes in a hanging bucket centrifuge. The cell pellet was then resuspended in complete medium and counted in *Neubauer* cell counting chamber. Viability of the cells was checked by trypan blue exclusion method. Appropriate number of cells was then sub cultured in fresh cell culture dishes with culture medium as per the experimental requirements.

Table 2.2: Cell types used in the present study

Cell Type	Name	Origin	Medium	Source
Immortalized Cell line	HaCaT	Human Keratinocytes	DMEM	NCCS, Pune
	HPLD	Human lung	Ham's F12	Dr. Lucy M Anderson, NIH, USA
	HEK	Human embryonic Kidney	DMEM	NCCS, Pune
Cancerous	U2OS	Human Osteosarcoma	DMEM	NCCS, Pune
	Saos 2	Human Osteosarcoma	DMEM	NCCS, Pune
	SiHa	Human Cervical Cancer	DMEM	NCCS, Pune
	HeLa	Human Cervical Cancer	DMEM	NCCS, Pune
	C33A	Human Cervical Cancer	DMEM	NCCS, Pune
	A549	Human Lung Adenocarcinima	DMEM	Dr. Lucy M Anderson, NCI, NIH, USA

2.2.1.2 Cell growth Assay

Cells were seeded in replicates of five @ $3X10^3$ cells per well in five different 96 well cell culture plates and grown in complete media. The method described earlier was slightly modified and followed (Gillies et al., 1986). After every 24h of seeding, one plate was stained with 0.2% crystal violet in 2 % ethanol for 15 minutes till 4^{th} day i.e. 96h. One plate was stained just after the cells get attached to use as 0h time point. Excess dye was removed from the plates by washing with ample amount of water. Crystal violet dye incorporated in the cells was extracted using 0.1% SDS solution by shaking for 10 minutes on a shaker. Absorbance of the extracted dye was then determined at 570 nm in a spectrophotometer. The experiment was repeated at least three times and the average absorbance was plotted for each time point to generate a growth curve.

2.2.1.3 Cell cycle analysis

The cells were collected at various time points by trypsinization, washed in phosphate buffered saline (PBS, pH 7.2) and fixed in chilled 70% methanol: ethanol (1:1) solution by

drop wise addition and kept at 4°C for 24h. Cells were then washed with PBS and stained with DNA staining solution at 37^0C in dark with intermittent shaking. The DNA content of cells was measured by flow cytometry using FACS-Aria (Beckton- Dickenson) at 695 ± 20 nm using a 655 long pass filter. The DNA content was then analysed by FACS Diva or FlowJo software to evaluate the various phases of cell cycle. The diploid 2N DNA content was referred as G1/G0 population and the 4N DNA content was referred as G2/M population. Cells with intermediary DNA content (between 2N - 4N) content were considered as S phase cells and those below 2N DNA content as sub G0 cells.

2.2.1.4 Extraction of total cellular protein

For preparation of cellular homogenate from adherent cell culture, the medium was first removed and cells were washed with ice cold 1X PBS. The cells were then scraped in 1X PBS and pellet down by gentle centrifugation (4000 rpm for 2 minutes) at 4^0C. Cell lysis buffer was then added to the cell pellets and lysis was allowed for 30 minutes on a rotor at 4°C. Post lysis, cells were centrifuged at 13000 rpm for 10min at 4°C. The pellet was discarded and supernatant was collected as cell homogenate.

2.2.1.5 Protein estimation

BCA (Bicinchoninic acid) method was used to determine the protein concentration in various samples. The Cu^{2+} ions from cupric sulphate (present in BCA reagent B) reagent are reduced to Cu^{+} by the protein in an alkaline medium. The cuprous ion (Cu^{+}) then combines with BCA (present in BCA reagent A) to give a purple colour whose intensity is proportional to the amount of protein present in the samples. This intensity is measured by colorimetry at 562 nm. BCA reagent was prepared by mixing reagent A with reagent B in a volume ratio of 50:1. A standard curve was generated using increasing concentrations of BSA (2-10µg) in a 25µl reaction, in a 96 well plate. Cell lysates were also diluted to same volume in parallel wells. 200µl of BCA reagent was then added to each well and incubated at 37^0C for 30 minutes. The absorbance readings were then taken in a spectrophotometer at 562 nm. Total protein was quantified by calculation of the slopes of regression lines of absorbance and BSA standards.

2.2.1.6 Immunoblotting

Equal amount of proteins were loaded on an appropriate percentage of denaturing SDS-PAGE gel. After completion of the run, the gel was over laid on a PVDF membrane cut to the size of gel and sandwiched between filter paper sheets and kept in the blotting cassette in the presence of transfer buffer. Finally the cassette was put in the mini transblot apparatus and blotting was done for 2-3 hours at a constant voltage of 80V at 4°C. For blocking the nonspecific sites membrane was incubated with blocking solution (5% non-fat milk solution in TBST) with gentle shaking for 1 hour at room temperature. Excess milk from the membrane was washed off with TBST and the membrane was incubated with primary antibody diluted in 1X TBST for 3 hours at room temperature or overnight at 4°C with shaking. After incubation the membrane was washed with TBST and incubated with appropriate secondary antibody (conjugated with horse-radish peroxidase) diluted in 5% fat free milk solution (in TBST) for 1h at room temperature. The blot was later washed thrice for 10min each with TBST and processed for the detection of protein signal using ECL-prime chemiluminescence detection reagent followed by detection of signal either on X-ray film or in a chemiluminescence detection system (Proteinsimple, California, USA).

2.2.1.7 Immunofluorescence Microscopy

Adherent cells growing either on cover slips or chamber slides were fixed with 4% paraformaldehyde for 10 min at room temperature. The cells were washed with PBS thrice for 5 min each and blocking was done in 2% BSA (prepared in PBS containing 0.3% Triton-X 100) for 1h. The cells were incubated with primary antibody (diluted in PBS containing 0.3% Triton-X 100) for 2h at room temperature or overnight at 4°C. The cells were washed with PBS thrice for 5 min each followed by incubation with Alexa Fluor 488- or 594-conjugated secondary (anti-mouse/rabbit) antibodies for 1h. Then the cells were mounted on microscopic slides using Vectashield mounting medium containing nuclear dye DAPI. Imaging was done by either the laser scanning confocal LSM 510 or LSM 750 (Carl Zeiss, Oberkochen, Germany) or fluorescence inverted (Olympus 1X51, Tokyo, Japan) microscopes.

2.2.1.8 Electrophoretic mobility shift assay

2.2.1.8.1 5' end labelling of the oligonucleotides

The oligonucleotides were labelled at their 5' end with ^{32}P using T4 polynucleotide kinase (T4 PNK) enzyme in a reaction given below.

Components		Volume (µl)	Final concentration
i.	Oligos (1pmole/µl)	2	2 pmoles
ii.	10X T4 PNK Buffer	5	1X
iii.	Sterile H_2O	37	
iv.	[γ-^{32}P] ATP (100µCi/µl)	2	200µCi (14.3 pmoles)
v.	T4 PNK	4	
Total		**50**	

The reaction was carried out by incubating at 37°C for 30 min. The reaction was stopped by adding 2µl of 0.5M EDTA, pH 8.0 and keeping on ice. A spin column was prepared using 1ml syringe and packed with sterile Sephadex G50 slurry and reaction mixture is applied on the top. The eluate is collected in different microcentrifuge tubes and radioactivity was counted using Geiger counter. The tube showing 7 to $9X10^6$ was used for experiment. The column containing the unincorporated [γ-^{32}P] ATP was discarded in radioactive waste bin. The radiolabelled oligonucleotides were annealed with their corresponding complementary unlabelled oligonucleotides. A 50 fold molar excess of the latter was used for annealing for conversion of labelled single strand to double strand. The tubes were kept in boiling waterbath for 3 min followed by room temperature for 30 min. The tubes were transferred to ice and the oligonucleotides were diluted to 4fmoles/µl using sterile H_2O.

2.2.1.8.2 Binding reaction

A binding reaction mixture was prepared by adding the following components to a microcentrifuge tube on ice.

Components		Volume (µl)
i.	^{32}P labelled double stranded oligonucleotides (4fmoles/µl)	4
ii.	10X Binding buffer	2
iii.	Poly dI-dC (1µg/ml)	2
iv.	10% NP40	2
v.	Sterile H_2O	6
vi.	Nuclear extract	4

The mixture is incubated in a water bath at 37°C for 15 min and afterwards transferred on ice and 4µl of DNA loading buffer is added. The samples were then run on a polyacrylamide gel electrophoresis which had been pre- run for 30 min. Electrophoresis was carried out at 4°C for 3h till the bromophenol blue migrated to 2cm above the bottom of gel. The gel was taken out and kept on Whatman filter paper sheet and covered by saran wrap followed by drying in a gel dryer at 80°C for 1h under suction. The dried gel was exposed to phosphoimager screen by keeping in phosphoimager cassette overnight.

2.2.1.9 Transient transfection in adherent cells

Transient transfection of plasmid DNA in cells was performed using Lipofectamine 2000 transfection reagent according to manufacturer's protocol. Briefly, 0.5 to 1 million cells were seeded in a 35mm tissue culture dish one day prior to transfection. For each 35mm dish, 4µg DNA was mixed in 250µl of Opti-MEM in one polypropylene tube. In another tube 10µl of Lipofectamine 2000 was diluted in 250µl Opti-MEM and incubated at room temperature for 5 minutes. DNA and Lipofectamine 2000 were mixed together and allowed to form complexes for 30 minutes at room temperature. Meanwhile, the adherent cells were washed twice with PBS and 1ml of Opti-MEM was added. 500µl of complexes were then added to each dish containing cells and medium. After 6-8 hrs, the medium containing complexes was removed and complete medium was added and transgene expression was evaluated 24-48 hrs after transfection. Since most of the experiments

involved use of GFP based vector system, the expression of the transgene was visualized under fluorescent microscope with excitation filter of 485± 20 nm.

2.2.2 Molecular techniques

2.2.2.1 Total RNA isolation from cultured cells

Total RNA was isolated by TRIzol method using the manufacturer's protocol. Briefly, medium was removed from culture dish and recommended amount of TRIzol was added directly on to the dish and kept at room temperature for 5 minutes for lysis of cells. The cellular homogenate was then transferred to a 1.5ml microcentrifuge tube. For each ml of TRIzol, 200µl of chloroform was added and tubes were shaken vigorously for 10 seconds to completely dissociate the nucleoprotein complexes, followed by vortexing for about 30 seconds. The mixture was kept for 3-5 minutes at room temperature and then centrifuged at maximum speed of 12,000 rpm for 10 minutes. The upper aqueous phase was transferred into a fresh micro centrifuge tube and RNA was precipitated by adding 500µl of iso-propanol. The RNA pellet was obtained by centrifugation at 12,000 rpm for 30 minutes at 4°C. The pellet was washed with 1ml of chilled 70% ethanol followed by centrifugation at 12,000 rpm for 5 minutes. The supernatant was removed and the pellet air-dried for about 5 minutes. The pellet was resuspended in 30-50µl RNase free deionised water and dissolved at 55ºC followed by quantification using nanodrop spectrophotometer for further use. The RNA integrity was checked by evaluating the 18S and 28S rRNA signals by running 1µl of total RNA on denaturing agarose gel stained with ethidium bromide.

2.2.2.2 Quantification of nucleic acids

The quantity and purity of nucleic acids was determined by measuring the absorbance at 260 and 280 nm. The concentration of nucleic acids was calculated by considering the OD (λ_{260}) = 1 corresponding to 50µg/ml DNA and 40 µg/ml of RNA. The purity of nucleic acids was checked by their A260/A280 ratio considering 1.8 for DNA and 2.0 for RNA. These measurements were done in NanoDrop 2000 UV-Vis Spectrophotometer.

2.2.2.3 RT-PCR (Reverse Transcriptase PCR)

2µg of total RNA was reverse-transcribed using SuperScript III Reverse Transcriptase which is a commercially available version of M-MLVRT with reduced RNase H activity and increased thermal stability. According to manufacturer's protocol 1µg of RNA, 1µl oligo (dT) (500ng), 1µl 10 mM dNTP and nuclease free water was added to a final volume of 13µl in a PCR tube. This mixture was then incubated at 65°C for 5 minutes in a thermo cycler and then quickly transferred to ice for 1minute. To this 4µl of 5X first strand buffer 1µl of 0.1M DTT and 1µl of RNaseOUT (40U/µl) were added. Then contents were then mixed and 1µl (200 units/µl) of SuperScript III RT was added. The mixture was then incubated at 50°C for 60 minutes in a thermo cycler. Lastly the reaction was stopped by incubating the mixture at 70°C for 15 minutes. The cDNA thus prepared was then used as a template for PCR.

2.2.2.4 Agarose Gel Electrophoresis

Agarose gels were prepared by boiling appropriate amount of agarose in TAE buffer. After dissolution, it was cooled and then poured in a casting tray containing a comb for desired number of wells. The gel was allowed to solidify and then shifted to horizontal electrophoresis tank containing TAE buffer. The DNA samples were mixed with appropriate volumes of 6X DNA loading dye, loaded on the gel and electrophoresed at appropriate voltage and current conditions (generally 80 V, 400 mA). The gel was stained in ethidium bromide solution (1 µg/ml) for 15-min at room temperature and visualised by fluorescence under UV-light in a UV-transilluminator.

2.2.2.5 DNA sequencing

Automated DNA sequencing on plasmid templates or on PCR products was carried out with dye terminator cycle sequencing kits from Perkin-Elmer on an automated sequencer (model 377, Applied Biosystems), following the manufacturer's instructions**.**

2.2.3 Recombinant DNA techniques

2.2.3.1 Isolation of plasmid DNA

Overnight grown bacterial culture (3 ml) was pellet down by centrifugation at 4ºC for10-min at 6000 rpm. The cells were re-suspended in 200µl of Resuspension solution (solution I). 400µl of freshly prepared Lysis solution (solution II) was then added and mixed by gently inverting the tubes for 4-6 times and allowed to lyse for 5 min at room temperature. The complete lysis was ascertained by uniformity and clarity of the contents. Subsequently, 400µl of Neutralization solution (solution III) was added and the tubes were inverted 4-6 times and gently for homogeneous mixing followed by incubation for 5 min on ice. After centrifuging at 12,000 rpm for 15-min, supernatant was decanted into a fresh tube, and 0.7 volume of iso-propanol was added. The precipitated nucleic acids were then recovered by centrifugation at 12,000 rpm for 30-min. The pellet was washed once with 70% ethanol, air-dried and re-suspended in 100µl of TE-buffer. It was treated with RNase at a concentration of 20µg/ml by incubating at 37ºC for 1hour. It was further extracted with an equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) mixture. After centrifugation, the clear supernatant was used for recovering the nucleic acids. The nucleic acids were precipitated with 2.5 volumes of ethanol in presence of 3 M sodium acetate. In case where high purity plasmid preparations are required (for transfection to cells) the plasmid isolation was carried out with the commercially available midiprep or miniprep kits following the manufacturer's instruction. Plasmids were observed on 1% agarose gel.

2.2.3.2 Restriction enzyme digestion

For restriction digestion (either single or double), 0.5 to 1µg of DNA was used in a reaction containing 2 to 5 units of commercially available restriction enzyme(s) and 5µl of the recommended buffer (supplied as 10X concentrations by the vendor) in a total reaction volume of 50µl. The reaction mixture was incubated for 2 h or overnight at 37°C. The digested DNA fragments were then visualised by ethidium bromide staining after electrophoresis on agarose gels. Commercially available DNA size markers were loaded along with the samples to ascertain or estimate the sizes of the digested fragments.

2.2.3.3 Purification of DNA by gel elution

Digested DNA fragments required for ligation were eluted from the agarose gel after electrophoresis. The gel was visualised over a UV illuminator and section of it containing the desired DNA fragment was carefully sliced out from the gel. The sliced agarose gel was then processed using commercially available gel elution kits for this purpose. The elution efficiency was checked by running a small aliquot of DNA sample on agarose gel.

2.2.3.4 Ligation of DNA

A total of 100-200ng of DNA was used in each ligation reaction. Vector to insert ratio of 1: 3 to 1: 5 was maintained. The reaction volume was generally maintained at 10µl containing 1µl of 10X ligation buffer (provided by the manufacturer) and 0.05 Weiss unit of T4-DNA ligase. The reaction was carried out at 16ºC for 14- to 16-hrs or at room temperature for 4 hours.

2.2.3.5 Transformation of ligated DNA

Of the ligation mixture, 2µl (of total volume of 10 µl reaction) was added to a tube of 100µl ultra competent DH5α bacterial cells and incubated in ice for 30 minutes. The tube was quickly transferred to a water bath maintained at 42°C to give a heat shock for 90 seconds and again quickly transferred to ice. 1ml of LB broth was added to the tube and then incubated at 37°C for 1 hour. The bacterial cells were then pellet down by centrifugation at 6000 rpm for 5 minutes and plated on LB agar containing appropriate antibiotic.

2.2.3.6 Preparation of Ultra competent cells

All the salts (10 mM PIPES, 15 mM $CaCl_2.2H_2O$, 250 mM KCl, 55 mM $MnCl_2.2H_2O$) except $MnCl_2$ were dissolved in water and pH was adjusted to 6.7 with 1N KOH. $MnCl_2$ was dissolved separately in water. $MnCl_2$ was added drop wise while stirring ($MnCl_2$ if added directly will give a brown colour to the solution and precipitates; hence it needs to be dissolved separately). Solution was then sterilized by filtering and stored. To prepare

competent cells pre-inoculum was prepared. A single bacterial colony was picked from LB agar plate that has been incubated for 16-20 hours at 37 °C and inoculated into 3 ml LB medium and incubated overnight at 37 °C temperature with 200 rpm shaking. 1% of this pre-inoculum was sub cultured in 100 ml LB-broth and incubated at 18 °C until OD 600 reached 0.5 - 0.6 (approx.). Culture was kept on ice for 10 min. with constant shaking. Cells were pelleted by centrifugation at 2000 x g /4°C /8 min. Pellet was resuspended in 40 ml of ice-cold Innoue buffer. Bacterial suspension was kept on ice for 30 min, re-spun at 2000 x g /4°C /8 min. Pellet was resuspended in 8 ml of TB buffer in which final concentration of DMSO was 7% and left on ice for 10 min. 100µl aliquots were made and snap frozen in liquid nitrogen and stored at -80 °C.

2.2.4 Quantification of blots and statistical analysis

The immunoblots were quantified by densitometry software ImageJ 1.17 developed by Wayne Rasband, NIH Bethesda, MD (http://rsb.info.nih.gov/nih-image).All experiments were done at least in triplicates and results were expressed as mean ± s.e.m. A two tailed Student's t-test was done in Graph pad to arrive at p values and differences were considered statistically significant when p-value was less than 0.05 (*$p \leq 0.05$), highly significant (**$p \leq 0.01$) and extremely significant (***$p \leq 0.001$).